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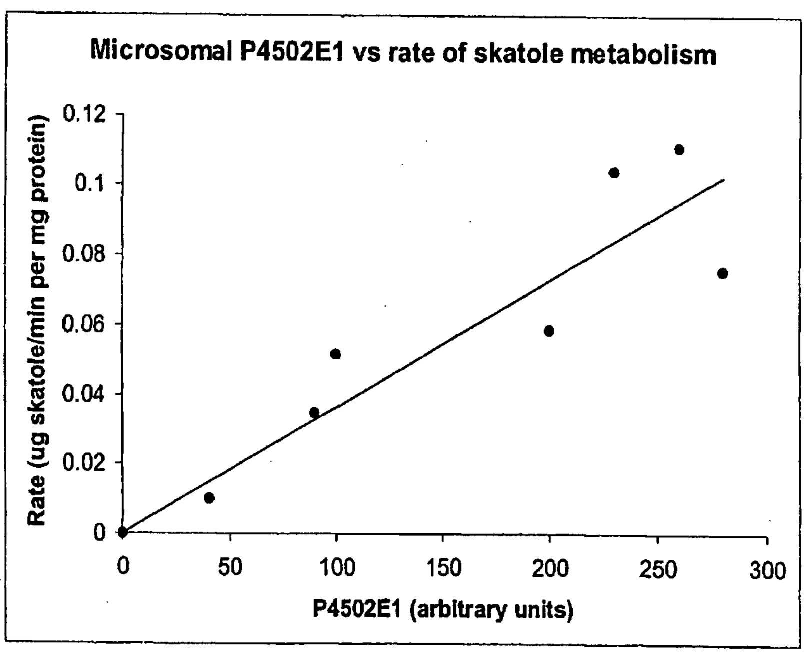

(57) Abstract: The present invention discloses a link between the level of cytochrome P450 isoform P4502E1 and the level of skatole. A polynucleotide encoding the sequence for P4502E1 and a polynucleotide encoding the sequence for P4502E1 and a polynucleotide encoding the sequence for P4502E1 for one pig with high skatole is provided. An assay to identify pigs with a genetic predisposition is also provided.

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DIAGNOSTIC ASSAY FOR BOAR TAINT

The present invention relates to genetic markers, which may include a functional mutation for pigs exhibiting desirable flavour properties. In particular, the present invention provides an assay to screen pigs for boar taint and its associated flavours. Generally pigs having low boar taint levels will be positively selected, but it is also possible to identify animals having unacceptably high boar taint levels.

Background

Boar taint - economic impacts

"Boar taint" is a strong perspiration-like, urine-like unpleasant odour given off upon heating or cooking of meat from some entire (uncastrated) male pigs. The off-odours and off-tastes, commonly known as "boar taint", are objectionable to consumers. In

the United States carcasses tainted by boar odour are either condemned or subject to restricted use by United States Department of Agriculture meat inspectors. EU law (Council Directive 91/497/EEC, which has been implemented in Britain through the Fresh Meat (Hygiene and Inspection) Regulations 1992)) states that animals over 80 kg carcass weight, excluding the head, should be screened for boar taint, but no method is specified.

The most effective method, to date, for preventing "boar taint" is to castrate (i.e., remove the testes of) young male pigs. Castration of young male pigs is widely practised in pig production systems in North America and Europe. However, as outlined below, there are production advantages of using entire male pigs. Entire male pigs are used extensively in pig production in the United Kingdom and also in Denmark, Australia and parts of Spain. Other measures taken to reduce the risk of boar taint include slaughtering entire male pigs at an earlier age than castrated males.

Pig production systems that involve castration of young male pigs suffer economic losses and other disadvantages. These economic losses are attributable to lost opportunities to access the superior performance, especially feed conversion, of intact males and the inferior nature of carcasses from castrates (barrows) (see for example: Allen, P., Riordan, P.B., Hanrahan, T.J. and Joseph, R.L. 1981.

Production and quality of boar and castrate bacon. *Irish J. Sci. Technol.* 5, 93-104; Wood, J.D. and Riley, J.E. 1982. Comparison of boars and castrates for bacon production. 1. Growth data, and carcass and joint composition. *Animal Production* 35, 55-63; Ellis, M., Smith, W.C., Clark, J.B.K. and Innes, N. 1983. A comparison of boars, gilts and castrates for bacon manufacture. 1. on farm performance, carcass and meat quality characteristics and weight loss in the preparation of sides for curing. *Animal Production* 37, 1-9). If the problem of boar taint were overcome, raising boars rather than castrates would have considerable economic advantages. Although boars and castrates gain weight at equivalent rates, boars produce carcasses containing 20-30% less fat. Boars also utilise feed more efficiently than barrows (10% less feed consumed per unit of body weight). Since feed represents the major cost in pig production, raising boars for pork would have significant economic advantages.

Castration not only produces animals with inferior carcass characteristics and a less efficient feed conversion, but is also bad for the pig's welfare. Adverse animal welfare considerations include the pain associated with castration, the loss of 'normal' behaviour and the risk of infection.

In conclusion, there is a need for methods to prevent or determine predisposition to boar taint, that do not require castration of young pigs.

Boar taint

Boar taint is associated with elevated levels of androstenone (5α-androst-16-en-3-one), indole and skatole (3-methyl-indole) See Patterson, R.L.S. (1968) 5α-androst-16-ene-3-one:-compound responsible for taint in boar fat. *J. Sci. Food Agric.* **19**: 31; Bonneau, M., Le Denmat, M., Vaudelet, J.C., Veloso Nunes, I.R. Mortensen, A.B. and Mortensen, H.P (1992) Contribution of fat androstenone and skatole to boar taint: II Eating quality of cooked ham. *Livest. Prod. Sci.* **32**, 81-88; see also Claus et al. 1994. Physiological aspects of androstenone and skatole formation in the boar - a review with experimental data. *Meat Science* **38**, 289-305.

Androstenone gives a urine or perspiration-like odour, whilst indole and skatole give a camphor-like odour. Levels of androstenone and skatole are each increased in non-castrated boars, although the reason for increased skatole levels has not been established. Additionally the formation of androstenone and skatole appears to be independent although the degradation of these compounds is currently believed to follow similar pathways and may each involve cytochrome P450s. There remains debate concerning the relative importance of androstenone and skatole in contributing to boar taint, and in certain studies emphasis has been placed onto androstenone (see WO 98/41861 and WO 99/18192). Methods that address the variation in levels of both

compounds would be particularly useful for breeding male slaughter pigs.

Skatole (3-methyl-indole) is produced by the breakdown of tryptophan by bacteria in the hindgut of pigs and other animals (see Moss et al., "Boar taint: the role of skatole", Meat Focus International, October 1992; and Babol et al., "Boar taint in entire male pigs", *EAAP Publication No 92*). Skatole is absorbed into the bloodstream and through the portal vein reaches the liver where it is metabolised. A number of isoforms of P450 exist but literature, and our own unpublished work suggest that metabolism of skatole depends on the P4502E1 isoform (Babol, J., Squires, E.J. and Lundstrom, K. (1998) Hepatic metabolism of skatole in pigs by cytochrome P4502E1 *J. Anim. Sci.* 76, 822-828 Squires, E.J. and Lundstrom, K. (1997) Relationship between cytochrome P4502E1 in liver and levels of skatole and its metabolites in intact male pigs. *J. Anim. Sci.* 75, 2506 -2511).

Skatole that is not metabolised for some reason is deposited in fatty tissues.

Methods for the identification and production of swine with reduced boar taint are described in WO 99/18192. The method of WO 99/18192 is concerned with androstenone production and in particular the predicted impact of specific natural or experimentally induced mutations or polymorphisms in

the porcine *CYP17* gene that encodes cytochrome P450c17. Cytochrome P450c17 is required for production of androstenone. No experimental data are provided to substantiate the claims - either of naturally occurring CYP17 variants in pigs or of experimentally induced mutations in the porcine *CYP17* gene. A method for determining predisposition to boar taint is disclosed in WO 98/41861. The method of WO 98/41861 is concerned with assaying for the presence of a low molecular weight isoform of cytochrome b5. Cytochrome b5 is involved with cytochrome P450c17 in the synthesis of androstenone. Although data relating levels of cytochrome b5 to levels of androstenone are presented, no evidence of a genetic component of the differences is presented. Neither the methods of WO 99/18192 nor WO 98/41861 address the contribution of skatole or indole. Skatole is critical to consideration of 'boar taint'. While about 25% of consumers are not able to smell androstenone (Claus, 1978. Der Geslechtsgeruch des Ebers aus der Sicht des Tierarztes, des Verbrauchers und der Tierproducktion. *Wien. Tierartztl Mschr* 65(12), 381-388) skatole is detected by all persons. Moreover, as skatole formation is not limited to the boar, an understanding of skatole production and clearance may be valuable in other meat species.

Previous research has suggested that part of the variation in boar taint or its component traits may be under genetic control.

Thus, Lundström and co-workers concluded from a study of skatole levels in pig selection lines that there is a genetic effect on skatole deposition which may be due to a recessive allele of a major gene (Lundström et al., 1994. Skatole levels in pigs selected for high lean tissue growth rate on different dietary protein levels. *Livest. Production Science* 38, 125-132).

Genetic selection

Selection against animals with a genetic predisposition to boar taint would be an attractive, cost-effective and humane solution to the problem of boar taint.

Skatole is believed to be the most important component of boar taint; boar taint being observed when not all skatole is degraded in the liver. A genetic component of boar taint could therefore be linked to a polymorphism in the cytochrome P450 isoform involved in skatole metabolism

We have now established that P4502E1 is the only P450 isoform involved in metabolism of skatole in the liver. Moreover we have confirmed that in Large White pigs, high liver levels of P4502E1 coincide with low levels of skatole in the backfat and vice versa. The mRNA levels of P4502E1 have been demonstrated to exhibit a similar relationship with skatole backfat levels.

Analysis has shown that animals with low skatole levels have a cDNA sequence similar to the one published by GenBank on the website *www.ncbi.nlm.nih.gov* under the reference Genbank/EMBL/DDBJ accession number: AB000885 (Kimura. M, Suzuki, H. and Hamasima, N., 1999. Cloning of the pig cytochrome P-450-j gene). However, the sequences of high skatole Large Whites are different from those of low skatole Large Whites in two locations. First, at position 648 of the database sequence Genbank/EMBL/DDBJ accession number: AB000885 low skatole pigs have a C, while high skatole pigs have a T; this does not change the amino acid sequence. Second, at position 1435 the G observed in low skatole pigs is changed to an A in high skatole pigs, resulting in an alanine residue in the expressed protein being changed to threonine. The complementary polymorphisms were detected in the complementary strand.

The experimental data herein presented provides evidence that:

i. skatole levels are inversely related to mRNA levels for P4502E1;

ii. polymorphisms exist at two locations in the coding sequence for P4502E1 and that this is associated with backfat skatole level;

iii. a polymorphism exists that changes the amino acid composition of P4502E1 and this is associated with backfat skatole levels; and

iv. in Meishans*Large White crosses a different factor than P4502E1 operates and causes levels of skatole in backfat to be high. Polymorphisms at location 648 and 1435 exist like in LW, but these are not associated with skatole levels.

In one aspect, the present invention provides a polynucleotide having a nucleotide sequence as set out in SEQ ID No 1 or SEQ ID No 3, their complementary sequences and the amino acid sequence derived therefrom. Further the present invention provides the use of these nucleotide sequences or portions thereof for use as genetic markers in screening pigs for boar taint phenotype. Preferably the genetic markers will include nucleotides 645 to 650 or nucleotides 1432 to 1438.

In a further aspect, the present invention provides an assay or method to identify pigs with a genetic predisposition that reduces the incidence of boar taint, wherein said assay comprises:

a) obtaining a DNA sample from a test pig;
b) analysing the sample to determine the allelic variant(s) at position 648 or 1435 or both;
c) using said results to select for animals of the preferred genotype.

The polymorphisms were found within the coding sequence of P4502E1 and the 1435 polymorphism actually alters the amino acid composition of the

expressed protein, and it is believed that one or both polymorphisms are responsible for boar taint phenotype via alterations to the function or expression of P4502E1. However, this has not been conclusively established and it remains possible that the polymorphisms described above (either separately or together) do not affect the function or expression of P4502E1 itself, but may be linked to the actual causative mutation elsewhere in the genome. In the latter case, the polymorphisms described herein will act as genetic markers. It is known to those skilled in the art that other genetic markers with a similar linkage may exist in the same region of the genome and they can be used instead. Linkage of these other genetic markers with skatole levels is part of the present invention.

Thus, the present invention provides a method to identify pigs with a genetic predisposition to a reduced incidence of boar taint, wherein said method comprises:

a) obtaining DNA samples from a population of pigs;
b) genotyping at least a sample of said population for at least one of (preferably both of) the polymorphism(s) described above;
c) measuring boar taint traits for at least a sample of said population;
d) correlating the presence of allelic variants of said polymorphism(s) with said traits;

e) obtaining a DNA sample from a test pig;

f) analysing the sample to determine the allelic variant(s) present at a said polymorphism; and

g) using the results obtained to select for animals of the preferred genotype.

The invention further relates to a method to approximate the actual boar taint level of a test pig wherein the method comprises:

a) obtaining a DNA sample from a test pig;

b) analysing the sample to determine the allelic variant at position 648 or 1435 or both;

c) using said results to approximate skatole levels in said test pig.

Preferably this method comprises:

a) obtaining DNA samples from a population of pigs;

b) genotyping at least a sample of said population for at least one (preferably both of) the polymorphism(s) occurring at positions 648 and 1435 of the P4502E1 coding sequence;

c) measuring boar taint traits for at least a sample of said population;

d) correlating the presence of allelic variants of said polymorphism(s) with said traits;

e) obtaining a DNA sample from a test pig;

f) analysing the sample to determine the allelic variant(s) present at a said polymorphism; and

g) using the results obtained to approximate skatole levels in said test pig.

Preferably the polymorphism is the allelic variant at position 648 or 1435 of the coding sequence for P4502E1 or a combination of the two. Other genetic markers that map within or close to P4502E1 may also be used, preferably in addition to the polymorphisms referred to above.

The animals shown to have marker genotypes or predicted genotypes indicative of a desirable boar taint predisposition (for example boars identified to have reduced boar taint), or the close relatives of such animals, can be used in a breading program, as breeding stock or for meat production.

In the assay or method of the present invention, the genomic DNA will be detected from a sample of porcine origin but the exact tissue forming the sample is not limited as long as it contains genomic DNA. Examples include body fluids such as blood, sperm, ascites and urine, tissue cells such as liver tissue, muscle, skin, hair follicles, fat and testicular tissue. The genomic DNA to be analysed can be prepared by extracting and purifying the DNA from such samples.

The method may be conducted *in vitro* or *in vivo* using a sample from a living animal or *post mortem* following the death of the animal being tested. If the assay is conducted *post mortem*, the information obtained may be of use for the siblings, parents or other close relatives of the animal.

Any suitable method may be used to determine the nucleotides at positions 648 and/or 1435. Mention may be made of the following suitable methods (although other methodologies may also be used). PCR-RFLP (polymerase chain reaction - restriction fragment length polymorphism), OLA (oligonucleotide ligation amplification), and methods for detecting single nucleotide polymorphisms (SNPs) including, but not limited to, hybridization-based methods, Third Wave's Invader technology and mass spectrometry-based methods.

Either of the polymorphisms in P4502E1 disclosed herein may prove to be the functional mutation or alternatively allow the isolation and characterisation of the functional mutation itself.

It remains possible that P4502E1 is not itself responsible for the observed variation in skatole levels, but merely contains a genetic marker linked with the functional mutation. Nonetheless (since the positioning of the mutation enables a search for linkage to the genes responsible for the trait) the present finding will facilitate identification of the

functional mutation. Once this mutation is located the option to manipulate the trait genes by transgenesis or to develop a further assay or method arises and forms part of the present invention.

The present invention will now be described in more detail by reference to the following, non-limiting, examples and figures in which:

Fig. 1 shows the rate of skatole metabolism in isolated liver microsomes as a function of microsome P4502E1 content confirming that the rate of skatole metabolism depends only on the content of P4502E1. Microsomes were isolated from seven different pigs and the P4502E1 content of each preparation was determined. A linear relationship was obtained (y) = 0.034 (x) + 0.004; correlation coefficient = 0.92).

Fig. 2a shows inhibition of microsomal skatole metabolism by allyl sulfide- a specific inhibitor of cytochrome P4502E1. Allyl sulphate was added to the incubation at a concentration of 1mM at zero time. Each point represents the mean ± S.E.M. for three independent experiments.

Fig. 2b shows that another specific inhibitor, chlorzoxazone (0.025 - 0.2 M), progressively inhibited metabolism of skatole, when measured after 40 minutes incubation.

Fig. 3 shows the relationship between backfat skatole and P4502E1 levels in liver microsomes. Microsomes were isolated from the livers of 12 Large White and 8 Meishan*Large White crosses pigs exhibiting a wide range of adipose tissue skatole levels. The results are from a number of different blots and the P4502E1 levels are normalised to 100% for one specific microsomal preparation, which was included on each blot.

Fig. 4 shows the correlation between hepatic P4502E1 mRNA levels and adipose tissue skatole content. RNA was extracted from liver samples from a number of pigs with different adipose tissue skatole levels and probed with P4502E1-specific DNA. The results are derived from several blots and are normalised to 100% for one RNA sample, which was present on all blots. Fig. 5 shows a part of the cDNA sequence coding for P4502E1 in pigs for one pig with high skatole and one with low skatole.

Fig. 6 shows the complete cDNA sequence (SEQ ID No 1) coding for P4502E1 for pigs with low skatole compared to the cDNA sequence Genbank/EMBL/DDBJ accession number: AB000885 published by GenBank.

Fig. 7 shows the amino acid sequence (SEQ ID No 2) derived from SEQ ID No 1 for pig with low skatole compared to the amino acid sequence coded by the cDNA sequence Genbank/EMBL/DDBJ accession number: AB000885 shown in Fig. 6.

Fig. 8 shows the cDNA sequence (SEQ ID No 3) coding for P4502E1 for one pig with high skatole.

Fig. 9 shows part of the amino acid sequence (SEQ ID No 4) derived from SEQ ID No 3 for pig with high skatole compared to amino acid sequence coded by the cDNA sequence Genbank/EMBL/DDBJ accession number: AB000885 shown in Fig. 8.

Fig. 10 shows part of the amino acid sequence (SEQ ID No 5) derived from SEQ ID No 3 for pig with high skatole compared to amino acid sequence coded by the cDNA sequence Genbank/EMBL/DDBJ accession number: AB000885 shown in Fig. 8.

Example 1

<u>Measurement of skatole metabolism by thin layer chromatography</u>

Samples of liver were obtained at 15 minutes post-mortem from intact male Large White pigs, frozen immediately in solid CO_2 and subsequently stored at -80°C for up to 2 months. Microsomes were isolated as described by Schenkman and Cinti, 1978 (Preparation of microsomes with calcium. *Methods Enzymol.* **52**, 83-89). A sample of pig liver (10 g) was homogenised with a "Polytron" homogeniser (Kinematica, Switzerland) for 1 minute in 40 ml of sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4), followed by centrifugation at 3,000 x g and 12,000 x g in order

to obtain the post-mitochondrial supernatant. Solid $CaCl_2$ was added at a final concentration of 8 mM and microsomes were sedimented at 25,000 x g for 15 minutes. The microsomal fraction was washed with KCl buffer (150 mM KCl, 10 mM Tris-HCl, pH 7.4). The pellet was suspended at a protein concentration of about 20 mg/ml in a medium containing 50 mM Tris-HCl, 10 mM KH_2PO_4, 0.1 mM EDTA, 20% glycerol and inhibitors of proteolytic enzymes (0.1 mM phenylmethylsulfonyl fluoride and 1 μg per ml pepstatin + antipain + leupeptin). Isolated microsomes were stored in liquid nitrogen for up to month. The rates of skatole metabolism in microsomes prepared from fresh and frozen samples of the same liver were found to be identical. The P4502E1 protein level in isolated microsomes was determined by Western Blotting using a commercial antibody. Briefly microsomal proteins were separated by SDS-PAGE (Cleveland, Fischer, Kirschner, Laemmli, 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J.Biol.Chem.* **252**, 1102-1106) and electro-blotted on to nitrocellulose. After successive incubations with anti-P4502E1 and peroxidase-labelled anti-rabbit IgG the blot was developed using an ECL procedure. The film was scanned and the 55kD bands were quantified using Imagequant programme (Molecular Dynamics). Isolated pig liver microsomes were incubated in 100 μl (total volume) of the medium, containing 50 mM Tris-HCl, 10 mM K_2HPO_4, 0.1 mM EDTA, 20% glycerol, pH 7.4 at 37°C in the presence of various concentrations

of skatole together with 1mM NADH plus 1mM NADPH as cofactors. The reaction was stopped by addition of 100 µl of ice-cold methanol. A zero time control was performed by adding methanol simultaneously with the microsomes. The mixture was vortexed for 1 minute and centrifuged for 10 minutes at 2,000 x g to precipitate the protein. The supernatant containing skatole was used for the skatole assays.

After centrifugation the supernatant was applied to a TLC plate in hexane:ether (4:1) and the plate was stained with Ehrlich reagent. The corresponding amount of pure skatole was run simultaneously. In such incubations two spots were obtained - a purple spot representing skatole and a pink spot at the origin representing any skatole metabolites which react with Ehrlich reagent. This latter spot was absent at zero time. During the progress of the reaction the intensity of the skatole spots decreased and that of the spots at the origin increased. Metabolites of skatole are not commercially available and therefore cannot be used as internal standards. Further, not all skatole metabolites react with Ehrlich reagent. Therefore in order to confirm that skatole was completely separated from its products by this procedure, samples from the same incubations were run on TLC and were also analysed by HRGC which allows complete separation and detection of all the skatole metabolites in the mixture.

Protein was determined by the Bradford method (Bradford, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 7;72:248-254) using bovine serum albumin as a standard.

Figure 1 shows skatole metabolism by isolated pig liver microsomes in the present of oxygen, NADH and NADPH. Measurements of skatole by the Thin Layer Chromatography (TLC) method coincided closely with High Resolution Gas Chromatography (HRGC) measurement of the same samples, thus validating the method.

Example 2

Determination of the P450 isoform involved in skatole metabolism.

The above experimental system described in Example 1 was used to investigate the involvement of cytochrome P4502E1 in skatole metabolism by pig liver microsomes. In initial experiments SKF-525A (0.1 mM), a general P450 inhibitor, completely inhibited skatole disappearance when measured over 15 minutes indicating that all skatole metabolism via the cytochrome P450 system (data not shown). Figure 2a shows that allyl sulphate, a specific P4502E1 inhibitor, completely inhibited skatole metabolism when added at over a 60 minute incubation period. Figure 2b shows that another specific inhibitor, chlorzoxazone (0.025 - 0.2 M), progressively

inhibited metabolism of skatole, when measured after 40 minutes incubation.

These results indicate that, in agreement with previous findings in the literature, skatole is metabolised via P4502E1.

Example 3

<u>Relationship between P4502E21 content, backfat skatole and rate of microsomal skatole metabolism.</u>

Liver samples were frozen in solid CO_2 within minutes of slaughter and kept at -80°C. Microsomes were isolated from frozen livers of selected pigs with various backfat skatole levels. Levels of P450 in the microsomes were derived from Western Blotting experiments using a commercial antibody stated to be specific for P4502E1. The initial rate of skatole metabolism was measured in the same preparations. Initial experiments showed that the rate of metabolism in microsomes from fresh liver was the same as those from liver frozen at -80°C for some weeks. Figure 3 shows that pigs with low backfat skatole levels all had high levels of P4502E1. Some pigs with high skatole backfat levels had low levels of P4502E1, but in a number of Meishan*Large White crosses pigs the P4502E1 level was only marginally reduced. In Large White pigs, the findings are similar to those of others (Squires and Lundstrom, 1997. Relationship between cytochrome P4502E1 in

liver and levels of skatole and its metabolites in intact male pigs. *J. Anim. Sci.* 75, 2506 -2511). In Meishan*Large White crosses pigs a different mechanism operates by which high skatole levels can exist with high P4502E1 levels.

However, the rate of skatole metabolism in microsomes varied by less than a factor of 1.5 when the P4502E1 level varied by a factor of 10 (Figure 4). This is consistent with the previous results of Babol, Squires and Lundstrom, 1998 (Relationship between oxidation and conjugation metabolism of skatole in pig liver and concentration of skatole in fat *J. Anim. Sci.* 76, 829-838). There was no correlation between rates of microsomal skatole metabolism and backfat skatole in the samples measured.

Example 4

Determination of P4502E1 mRNA in liver by Northern blotting

A 375bp cDNA probe corresponding to bases 507 - 881 of the pig P4502E1 sequence Genbank/EMBL/DDBJ accession numbers: AB000885 was generated by Polymerase chain reaction (PCR) with pig liver cDNA as template; the identity of the probe was checked by DNA sequencing. The probe was labelled with α-^{32}P dCTP using the Boehringer Hi-Prime kit. Total RNA was extracted from frozen liver using Tri-Reagent (Sigma) and 20 µg RNA was separated on an agarose gel

as described by Maniatis et al., Sambrook et al. (in Sambrook, Fritsch, Maniatis, 1989. Analysis of RNA. *Molecular cloning*. A laboratory manual, 1, 7.37-7.57). After pre-hybridisation the blot was hybridised overnight at 42°C and washed at 42°C in SSPE followed by 2 washes in 50°C in SSPE/SDS 0.1%. After autoradiography, the bands were quantified by scanning using the Imagequant programme.

Figure 4 shows that there was an inverse correlation between backfat skatole and P4502E1 mRNA expression in Large White pigs. This confirms that P4502E1 is involved in skatole metabolism.

Example 5

DNA sequencing

We have used three Large White pigs with very low skatole (0.019, 0.026 and 0.118 µg/mg backfat), three Large White pigs with high skatole (1.309, 0.740 and 0.400 µg/mg backfat) and one Meishan*Large White crosses pig with high skatole (0.914 µg/mg backfat).

RNA was isolated from each liver using Tri-Reagent. First strand DNA was synthesised using reverse transcriptase and oligo dT priming. PCR primers were designed corresponding to various locations on the database sequence of pig P4502E1. The DNA was used as a template for PCR. The single PCR product of the

correct size was extracted, ligated into the pGem vector and used to transform E. coli (XL-1Blue). The insert size was checked after double digestion with EcoR1 and the insert was sequenced in the plasmid using M13 forward and reverse primers. The primers used were:

Forward primer: 5'CATCTCCATCTGGAAGCACATC 3'
Reserve primer: 5' ACACTTGTGAGCGGGGAATG 3'

Figure 5 shows cDNA sequences for the five animals and the corresponding sequence on the database Genbank/EMBL/DDBJ accession number: AB 000885 showing differences between the sequence entry (Genbank/EMBL/DDBJ accession number: AB000885) and low skatole pigs on one hand and high skatole pigs on the other at locations 648 and 1435.

Additionally, differences have been found between the coding sequence of all seven pigs and the sequence Genbank/EMBL/DDBJ accession number: AB00085. In addition to the differences appearing at the extremities of the cDNA sequence, two differences in the coding region at positions 1087 and 1180-1181 have been found.

Studies carried on two additional high skatole Meishan*Large White crosses pigs have shown that amongst the three high skatole pigs analysed two have the same polymorphisms seen in the high skatole Large White pigs while the other does not. These findings

are in line with the fact that we find that in Meishan*Large White crosses different or additional mechanisms may operate.

Example 6

PCR-RFLP assay for polymorphisms at nt 1435

A PCR-RFLP assay for the polymorphism at the nucleotide (nt) corresponding to nt 1435 in the cDNA sequence has been developed. The sequence CGCG at nt 1434-1437 in the cDNA sequence corresponds to the cleavage site for the restriction endonuclease BstUI. When this sequence is CACG the sequence is not recognised or cleaved by BstUI. We predicted the location of the exon-intron boundaries in the cDNA sequence by comparison with the human *CYP2E* gene (EMBL/Genbank accession number: J02843). Oligonucleotide primers with the following sequences were designed for the amplification of a 172 bp fragment including the polymorphic nucleotide from genomic DNA.

Forward primer: 5'-GGGTGTGTGTCGGAGAGG-3'
Reverse primer: 5'-CGGGGAATGACACAGAGTTT-3'

Amplification of the 172bp fragment from genomic DNA was effected by the Polymerase Chain Reaction (PCR) in a total volume of 50 microlitres. PCR reactions contained 100 ng genomic DNA, 1 x PCR buffer (Roche), 1.5 mM $MgCl_2$, 100 μM dNTPs, 500 nM each primer, 1

unit *Taq* DNA polymerase. PCR conditions were 94°C for 3mins, then 35 cycles of 94°C for 30 seconds, 56°C for 45 seconds and 72°C for 1 min. PCR products were digested by adding 10 units of BstUI to the reaction mix and incubating at 60°C overnight. The digested PCR products were fractionated by electrophoresis through a 2.5% Metaphor™ (Flowgen) agarose gel. Where nt 1435 is a cytosine (C) the 172 bp fragment is cleaved by BstUI to yield products of 142 and 30 bps. Where nt 1435 is an adenine (A) the 172 bp fragment is not cleaved by BstUI. In samples from animals with one C and one A allele (i.e. heterozygotes) fragments of 172, 142 and 30 bp are observed.

CLAIMS

1. A polynucleotide having a nucleotide sequence as set out in SEQ ID No 1 or 3 or a complementary sequence thereof.

2. An assay to identify pigs with a genetic predisposition to boar taint wherein said assay comprises:
   a) obtaining a DNA sample from a test pig;
   b) analysing the sample to determine the allelic variant at position 648 or 1435 or both;
   c) using said results to select for animals of the preferred genotype.

3. The assay as claimed in Claim 2 wherein the allelic variation at position 648 is determined.

4. The assay as claimed in Claim 2 wherein the allelic variation at position 1435 is determined.

5. The assay as claimed in Claim 2 wherein other genetic markers that map within or close to P4502E1 are used in addition to or instead of determining the allelic variant at position 648 or 1435 or both.

6. The assay as claimed in any one of Claims 2 to 5 wherein the nucleotides at positions 648 or

1145 are determined by PCR-RFLP, OLA, mass spectrometry based methods, methods for detecting single nucleotide polymorphisms (SNPs) like hybridization-based methods, or Third Wave's Invader technology.

7. The assay as claimed in any one of Claims 1 to 6 wherein the method is conducted on the test pig post mortem.

8. A method to identify pigs with a genetic predisposition to boar taint comprising:
   a) obtaining a DNA sample from a test pig;
   b) analysing the sample to determine the allelic variant at position 648 or 1435 or both;
   c) using said results to select for animals of the preferred genotype.

9. A method to identify pigs with a genetic predisposition to a reduced incidence of boar taint, wherein said method comprises:

   a) obtaining DNA samples from a population of pigs;
   b) genotyping at least a sample of said population for at least one (preferably both of) the polymorphism(s) occurring at positions 648 and 1435 of the P4502E1 coding sequence;

c) measuring boar taint traits for at least a sample of said population;

d) correlating the presence of allelic variants of said polymorphism(s) with said traits;

e) obtaining a DNA sample from a test pig;

f) analysing the sample to determine the allelic variant(s) present at a said polymorphism; and

g) using the results obtained to select for animals of the preferred genotype.

10. A method as claimed in Claim 9 wherein other genetic markers that map within or close to P4502E1 are used in addition to or instead of the polymorphisms of step b).

11. A method of selecting an animal for use in a breeding program, said method comprising using said animal as a test animal in step e) of Claim 9.

12. A method as claimed in Claim 9 wherein other genetic markers that map within or close to P4502E1 are used in addition to or instead of the polymorphisms occurring at positions 648 and 1435 of the P4502E1 coding sequence.

13. A method to approximate the actual boar taint level of a test pig wherein said method comprises:

a) obtaining a DNA sample from a test pig;

b) analysing the sample to determine the allelic variant at position 648 or 1435 or both;

c) using said results to approximate skatole levels in said test pig.

14. A method to approximate the actual boar taint level of a test pig, wherein said method comprises:

a) obtaining DNA samples from a population of pigs;

b) genotyping at least a sample of said population for at least one (preferably both of) the polymorphism(s) occurring at positions 648 and 1435 of the P4502E1 coding sequence;

c) measuring boar taint traits for at least a sample of said population;

d) correlating the presence of allelic variants of said polymorphism(s) with said traits;

e) obtaining a DNA sample from a test pig;

f) analysing the sample to determine the allelic variant(s) present at a said polymorphism; and

g) using the results obtained to approximate skatole levels in said test pig.

15. The method as claimed in any one of Claims 8 to 14 wherein the nucleotides at positions 648 or 1145 are determined by PCR-RFLP, OLA, mass spectrometry based methods, methods for

detecting single nucleotide polymorphisms (SNPs) like hybridization-based methods, or Third Wave's Invader technology.

16. The method as claimed in any one of Claims 8 to 15 wherein the method is conducted on the test pig post mortem.

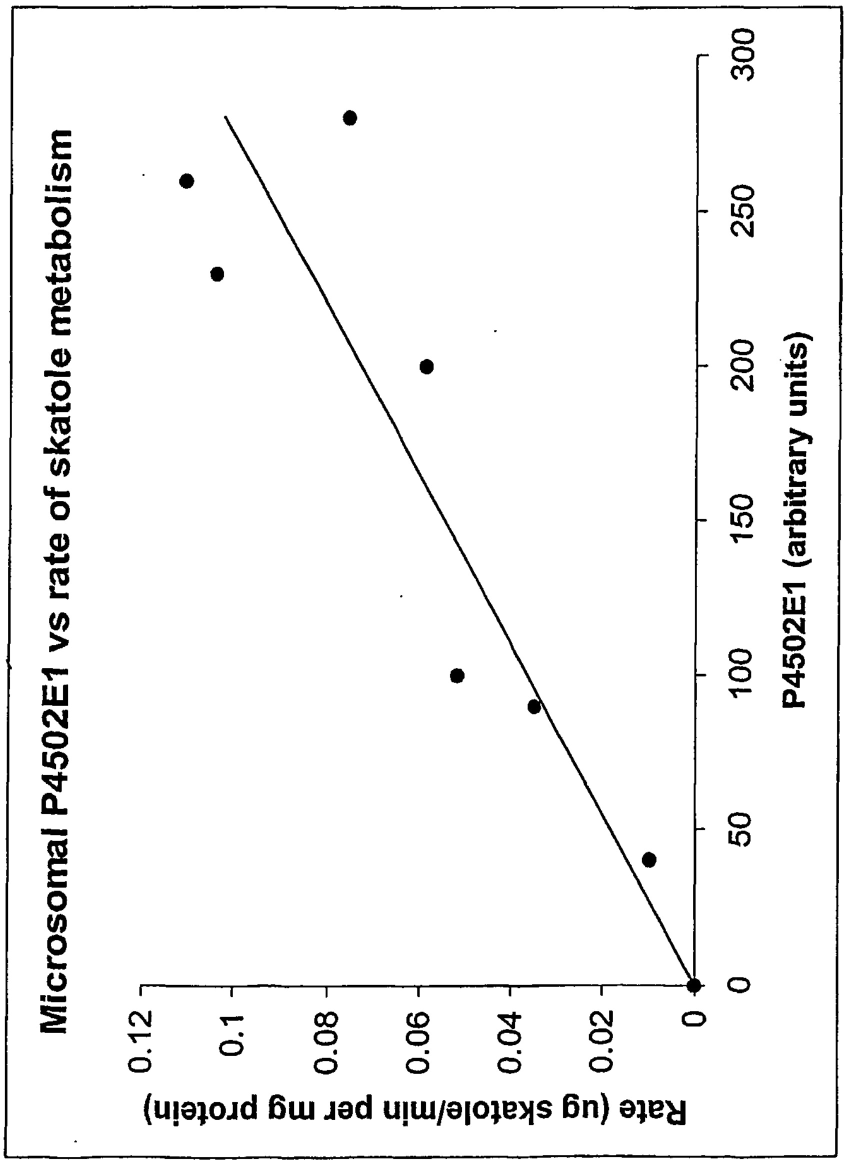
Microsomal P4502E1 vs rate of skatole metabolism
Rate (ug skatole/min per mg protein)
0.12
0.1
0.08
0.06
0.04
0.02
0
0
50
100
150
200
250
300
P4502E1 (arbitrary units)

Fig. 1

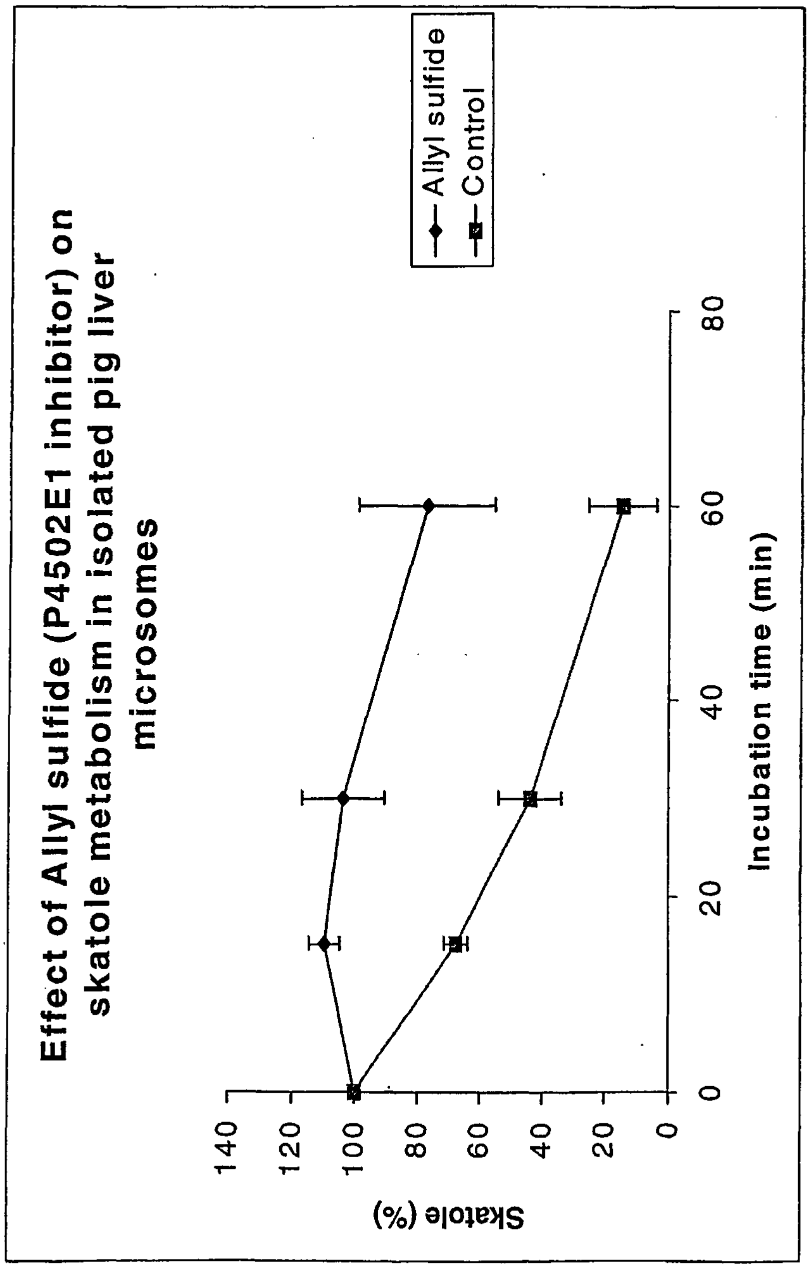
Effect of Allyl sulfide (P4502E1 inhibitor) on skatole metabolism in isolated pig liver microsomes
Allyl sulfide
Control
Skatole (%)
140
120
100
80
60
40
20
0
0
20
40
60
80
Incubation time (min)

Fig. 2a

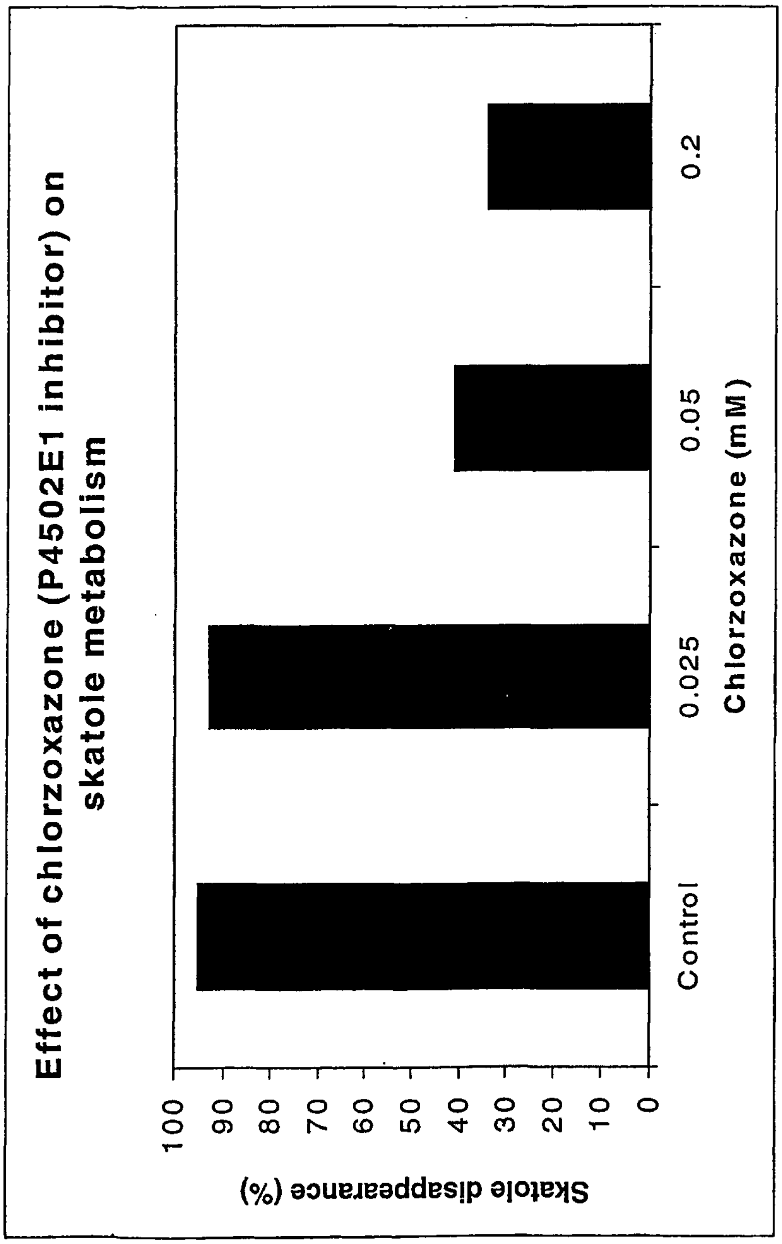
Effect of chlorzoxazone (P4502E1 inhibitor) on skatole metabolism
Skatole disappearance (%)
100
90
80
70
60
50
40
30
20
10
0
Control
0.025
0.05
0.2
Chlorzoxazone (mM)

Fig. 2b

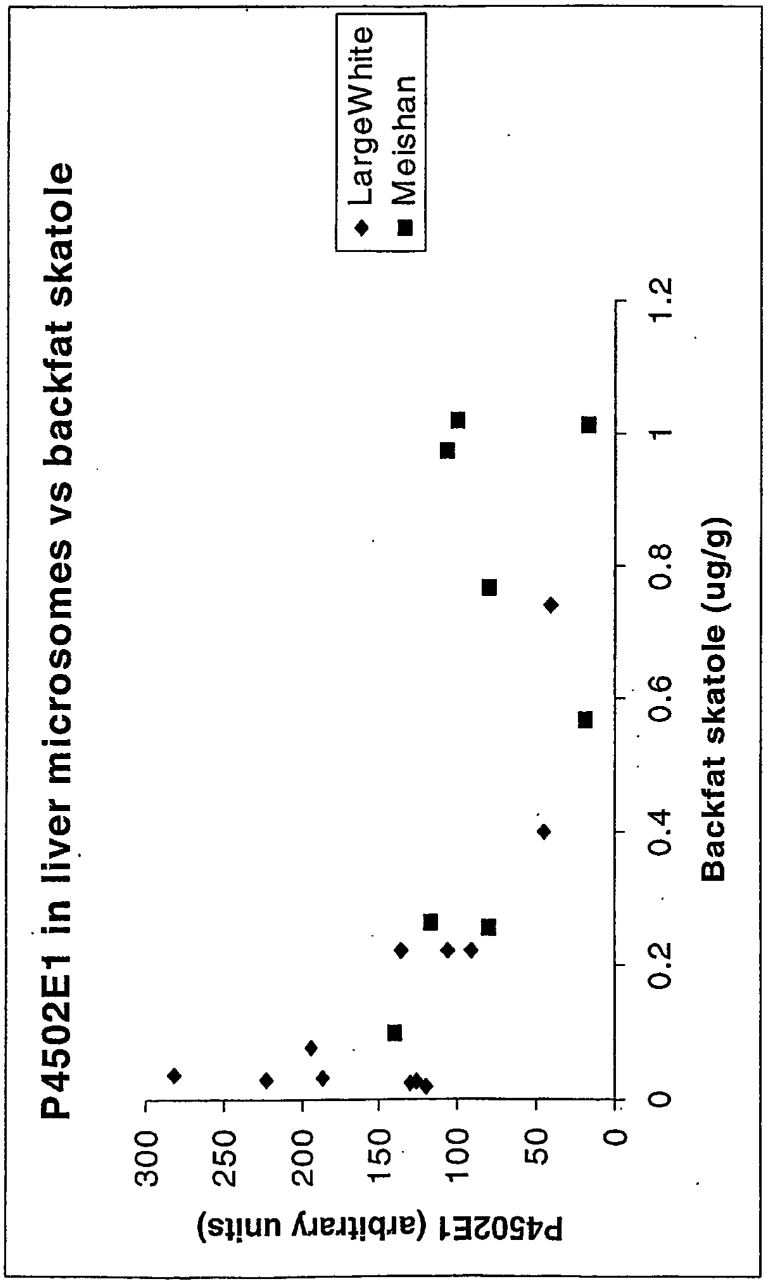
P4502E1 in liver microsomes vs backfat skatole
LargeWhite
Meishan
P4502E1 (arbitrary units)
300
250
200
150
100
50
0
0
0.2
0.4
0.6
0.8
1
1.2
Backfat skatole (ug/g)

Fig. 3

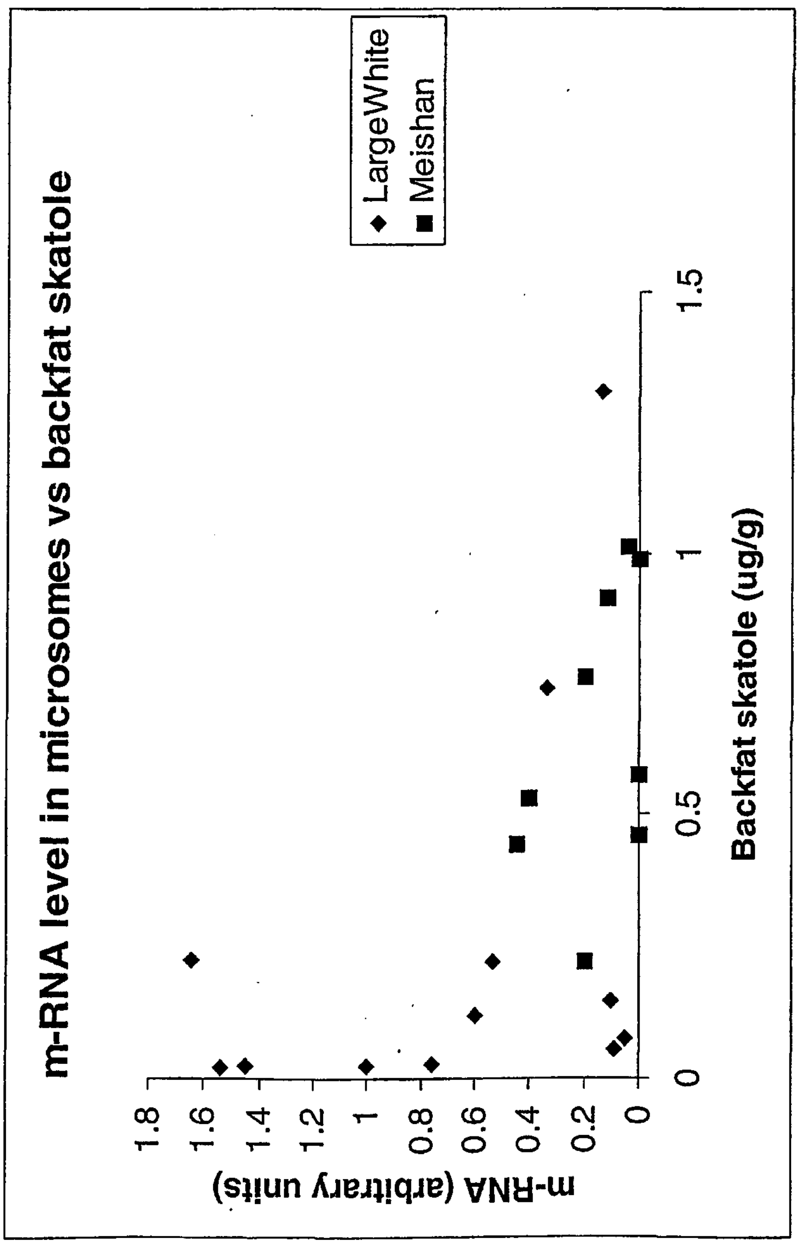
m-RNA level in microsomes vs backfat skatole
m-RNA (arbitrary units)
Backfat skatole (ug/g)
LargeWhite
Meishan
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
0.5
1
1.5

Fig. 4

6/14

Partial sequence alignment of P4502E1 from Large White high skatole (1.309µg/g) pig (capitals) v. Large White low skatole (0.019µg/g) pig (small letters). Note polymorphisms at 648 and 1435.

```
     TCTCCGACATCCTCTTCCGCCAGCACTTTGACTACAATGACAAGACCTGT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
551  tctccgacatcctcttccgccagcactttgactacaatgacaagacctgt 600
              .         .         .         .         .
     CTGAGGTTAATGAGCATGTTCAATGAGAACTTCTACCTGCTCAGTACTGG
     ||||||||||||||||||||||||||||||||||||||||||||||| ||
601  ctgaggttaatgagcatgttcaatgagaacttctacctgctcagtaccgg 650
              .         .         .         .         .
     CTGGATCCAGCTTTATAATAATTTCTCAGGCTATCTACGCTACCTGCCTG
     ||||||||||||||||||||||||||||||||||||||||||||||||||
651  ctggatccagctttataataatttctcaggctatctacgctacctgcctg 700
              .         .         .         .         .
     GAAGCCATAGGAAACTAATGAAAAATATATCTGAAATAAAAGATTATGCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
701  gaagccataggaaactaatgaaaaatatatctgaaataaaagattatgct 750
...........................................................
     CTGTTCCTGTTCATGGCTGCCATCTTGCAGCACTTTAACTTGAAATCTCT
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1351 ctgttcctgttcatggctgccatcttgcagcactttaacttgaaatctct
1400
              .         .         .         .         .
     TGTTGACCCCAAGGATATCGACCTCAGCCCCATCACGATTGGGTTTGCCA
     |||||||||||||||||||||||||||||||||| |||||||||||||||
1401 tgttgaccccaaggatatcgacctcagccccatcgcgattgggtttgcca
1450
              .         .         .         .         .
     AGATTCCCCCCCATTACAAACTCTGTGTCATTCCCCGCTCACAAGTGT
     ||||||||||||||||||||||||||||||||||||||||||||||||
1451 agattccccccattacaaactctgtgtcattccccgctcacaagtgt 1500
```

Fig. 5

```
1              GCACACATTGAAAGATCCCCTGAAGGAGCC 50    6 SEQ ID No. 1
                                  |  | |  |||
1 ..................................cggcacgaggcc 12   - AB000885

51  ATGACTGCCCTGGGCATCACGGTGGCCCTGCTGGTGTGGTTGGTCACCCT 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
13  atgactgccctgggcatcacggtggccctgctggtgtggttggtcaccct 62

101 GCTGCTCATCTCCATCTGGAAGCACATCCACAGTAGCTGGAAACTTCCCC 150
    ||||||||||||||||||||||||||||||||||||||||||||||||||
63  gctgctcatctccatctggaagcacatccacagtagctggaaacttcccc 112

151 CTGGCCCTTTCCCACTGCCCATCGTTGGGAACATTTTCCAGTTGGACCTT 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
113 ctggccctttcccactgcccatcgttgggaacattttccagttggacctt 162

201 AAGAATATTCCCAAATCCTTCACCATGCTGGCAGAGCGTTACGGGCCGGT 250
    ||||||||||||||||||||||||||||||||||||||||||||||||||
163 aagaatattcccaaatccttcaccatgctggcagagcgttacgggccggt 212

251 GTTCACTGTGTACCTGGGTTCGCGGCGCATTGTGGTCCTGCACGGCTACA 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
213 gttcactgtgtacctgggttcgcggcgcattgtggtcctgcacggctaca 262

301 AGGCCGTGAAGGAGGTCTTGCTCCACTACAAGAATGAGTTCTCTGGCAGA 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
263 aggccgtgaaggaggtcttgctccactacaagaatgagttctctggcaga 312

351 GGGGAAATCCCCACGTTCCAAGTGCACAAGGACAAAGGGGTCATTTTCAA 400
    ||||||||||||||||||||||||||||||||||||||||||||||||||
313 ggggaaatccccacgttccaagtgcacaaggacaaaggggtcattttcaa 362

401 TAATGGACCAACCTGGCGGGACACTCGGCGGTTCTCCCTCACCACCCTCC 450
    ||||||||||||||||||||||||||||||||||||||||||||||||||
363 taatggaccaacctggcgggacactcggcggttctccctcaccaccctcc 412

451 GTGACTTCGGGATGGGGAAACAGGGCAATGAGCAGCGGATCCAGAGGGAG 500
    ||||||||||||||||||||||||||||||||||||||||||||||||||
413 gtgacttcgggatggggaaacagggcaatgagcagcggatccagagggag 462

501 GCCCACTTCCTGCTGGAGGCACTCAGGAAGACCCATGGCCAGCCCTTTGA 550
    ||||||||||||||||||||||||||||||||||||||||||||||||||
463 gcccacttcctgctggaggcactcaggaagacccatggccagccctttga 512

551 TCCCACCTTCCTCATCGGCTGCGCACCCTGCAATGTCATCTCCGACATCC 600
    ||||||||||||||||||||||||||||||||||||||||||||||||||
513 tcccaccttcctcatcggctgcgcaccctgcaatgtcatctccgacatcc 562

601 TCTTCCGCCAGCACTTTGACTACAATGACAAGACCTGTCTGAGGTTAATG 650
    ||||||||||||||||||||||||||||||||||||||||||||||||||
563 tcttccgccagcactttgactacaatgacaagacctgtctgaggttaatg 612
:al.pair (40%)
```

Fig. 6

```
 651 AGCATGTTCAATGAGAACTTCTACCTGCTCAGTACCGGCTGGATCCAGCT 700
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 613 agcatgttcaatgagaacttctacctgctcagtaccggctggatccagct 662
                                     648

 701 TTATAATAATTTCTCAGGCTATCTACGCTACCTGCCTGGAAGCCATAGGA 750
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 663 ttataataatttctcaggctatctacgctacctgcctggaagccatagga 712

 751 AACTAATGAAAAATATATCTGAAATAAAAGATTATGCTTTAGAAAGAGTG 800
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 713 aactaatgaaaaatatatctgaaataaaagattatgctttagaaagagtg 762

 801 AAGGACCACCGGGATTCACTGGAGCCCAGCTGTCCTCGAGATTTCACTGA 850
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 763 aaggaccaccgggattcactggagcccagctgtcctcgagatttcactga 812

 851 CACCCTGCTGATGGAAATGGAGAAGGAAAAATACAGTGCAGAACCTATAT 900
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 813 caccctgctgatggaaatggagaaggaaaaatacagtgcagaacctatat 862

 901 ACACCTTGGACAACATTGCCGTGACCGTGGCCGACATGTTCTTTGCGGGG 950
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 863 acaccttggacaacattgccgtgaccgtggccgacatgttctttgcgggg 912

 951 ACAGAGACCACCAGCACCACCCTGAGATACGGGCTCCTAATTCTCATGAA 1000
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 913 acagagaccaccagcaccaccctgagatacgggctcctaattctcatgaa 962

1001 ATACCCAGAGGTTGAAGAGAAACTTCATGAAGAAATTGACAGGGTCATTG 1050
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 963 atacccagaggttgaagagaaacttcatgaagaaattgacagggtcattg 1012

1051 GTCCAAACAGAATCCCTGCCATCAAGGACAGGCTGGACATGCCCTACCTG 1100
     |||||||||||||||||||||||||||||||||||| |||||||||||||
1013 gtccaaacagaatccctgccatcaaggacaggctggtcatgccctacctg 1062

1101 GATGCCGTGGTACATGAGATTCAGCGATTCATCGACCTCATTCCCTCCAA 1150
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1063 gatgccgtggtacatgagattcagcgattcatcgacctcattccctccaa 1112

1151 CCTGCCACATGAAGCAACCCGGGACACAGTATTCAGAGACTACATCATCC 1200
     ||||||||||||||||||||||||||||||  ||||||||||||||||||
1113 cctgccacatgaagcaacccgggacacagatttcagagactacatcatcc 1162

1201 CCAAGGGCACAGTGGTAATTCCGACACTGGACTCCGTCTTATATGACAGC 1250
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1163 ccaagggcacagtggtaattccgacactggactccgtcttatatgacagc 1212

1251 CAAGAATTCCCTGAGCCGGAGAAGTTTAAGCCAGAGCACTTTCTGAATGA 1300
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1213 caagaattccctgagccggagaagtttaagccagagcactttctgaatga 1262
46total.pair (749)
```

Fig. 6 (Continued)

```
1301 AAACGGAAAGTTCAAGTACAGTGATCATTTCAAGGCATTTTCCGCAGGAA 1350
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1263 aaacggaaagttcaagtacagtgatcatttcaaggcattttccgcaggaa 1312

1351 AGCGGGTGTGTGTCGGAGAGGGCCTGGCTCGCATGGAACTGTTCCTGTTC 1400
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1313 agcgggtgtgtgtcggagagggcctggctcgcatggaactgttcctgttc 1362

1401 ATGGCTGCCATCTTGCAGCACTTTAACTTGAAATCTCTTGTTGACCCCAA 1450
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1363 atggctgccatcttgcagcactttaacttgaaatctcttgttgaccccaa 1412

1451 GGATATCGACCTCAGCCCCATCGCGATTGGGTTTGCCAAGATTCCCCCCC 1500
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1413 ggatatcgacctcagccccatcgcgattgggtttgccaagattccccccc 1462
                            1435

1501 ATTACAAACTCTGTGTCATTCCCCGCTCACAAGTGTGAGGGAGATGTGCT 1550
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1463 attacaaactctgtgtcattccccgctcacaagtgtgagggagatgtgct 1512

1551 CTAAAGGCCCTGGTTCCTTGATGCTGACCTGGAGGCCTCCTGTCCCCAGT 1600
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1513 ctaaaggccctggttccttgatgctgacctggaggcctcctgtccccagt 1562

1601 GTCCCCACAGGGAGCGCAGCCCGGGCTCCATAGGAAATCAAATGGGCCAG 1650
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1563 gtccccacagggagcgcagcccgggctccataggaaatcaaatgggccag 1612

1651 TGAAGCTGCTTCCAGCCCACATCCTTCAGATAGAATTTGAAAGCAAAGTC 1700
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1613 tgaagctgcttccagcccacatccttcagatagaatttgaaagcaaagtc 1662

1701 CAAAAAAGATTTTGTACAATCAATTAAAGTAAGTAAAGCCAAAAAAAAAA 1750
     ||||||||||||||||||||||||||||||||||||||||
1663 caaaaaagattttgtacaatcaattaaagtaagtaaagct.......... 1702
```

spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%
spice 18%

Fig. 6 (End)

```
  1 ....MTALGITVALLVWLVTLLLISIWKHIHSSWKLPPGPFPLPIVGNIF  46   - SEQ ID No. 2
        ||||||||||||||||||||||||||||||||||||||||||||||
  1 RHEAMTALGITVALLVWLVTLLLISIWKHIHSSWKLPPGPFPLPIVGNIF  50   - AB000885

 47 QLDLKNIPKSFTMLAERYGPVFTVYLGSRRIVVLHGYKAVKEVLLHYKNE  96
    ||||||||||||||||||||||||||||||||||||||||||||||||||
 51 QLDLKNIPKSFTMLAERYGPVFTVYLGSRRIVVLHGYKAVKEVLLHYKNE 100

 97 FSGRGEIPTFQVHKDKGVIFNNGPTWRDTRRFSLTTLRDFGMGKQGNEQR 146
    ||||||||||||||||||||||||||||||||||||||||||||||||||
101 FSGRGEIPTFQVHKDKGVIFNNGPTWRDTRRFSLTTLRDFGMGKQGNEQR 150

147 IQREAHFLLEALRKTHGQPFDPTFLIGCAPCNVISDILFRQHFDYNDKTC 196
    ||||||||||||||||||||||||||||||||||||||||||||||||||
151 IQREAHFLLEALRKTHGQPFDPTFLIGCAPCNVISDILFRQHFDYNDKTC 200

197 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLMKNISEIKDYA 246
    ||||||||||||||||||||||||||||||||||||||||||||||||||
201 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLMKNISEIKDYA 250

247 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSAEPIYTLDNIAVTVADM 296
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSAEPIYTLDNIAVTVADM 300

297 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLD 346
    |||||||||||||||||||||||||||||||||||||||||||||||||
301 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLV 350

347 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTVVIPTLDSV 396
    |||||||||||||||||||||||||||||| |||||||||||||||||||
351 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTDFRDYIIPKGTVVIPTLDSV 400

397 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 446
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 450

447 LFLFMAAILQHFNLKSLVDPKDIDLSPIAIGFAKIPPHYKLCVIPRSQV* 496
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 LFLFMAAILQHFNLKSLVDPKDIDLSPIAIGFAKIPPHYKLCVIPRSQV* 500

497 GRCALKALVP*C*PGGLLSPVSPQGAQPGLHRKSNGPVKLLPAHILQIEF 546
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 GRCALKALVP*C*PGGLLSPVSPQGAQPGLHRKSNGPVKLLPAHILQIEF 550

547 ESKV............. 550
    ||||
551 ESKVQKRFCTIN*SK*S 567
spice 23%
spice 23%
spice 23%
```

Fig. 7

```
1                                GCACACATTG AAAGATCCCC TGAAGGAGCC   - SEQ ID No3
51    ATGACTGCCC TGGGCATCAC GGTGGCCCTG CTGGTGTGGT TGGTCACCCT
101   GCTGCTCATC TCCATCTGGA AGCACATCCA CAGTAGCTGG AAACTTCCCC
151   CTGGCCCTTT CCCACTGCCC ATCGTTGGGA ACATTTTCCA GTTGGACCTT
201   AAGAATATTC CCAAATCCTT CACCATGCTG GCAGAGCGTT ACGGGCCGGT
251   GTTCACTGTG TACCTGGGTT CGCGGCGCAT TGTGGTCCTG CACGGCTACA
301   AGGCCGTGAA GGAGGTCTTG CTCCACTACA AGAATGAGTT CTCTGGCAGA
351   GGGGAAATCC CCACGTTCCA AGTGCACAAG GACAAAGGGG TCATTTTCAA
401   TAATGGACCA ACCTGGCGGG ACACTCGGCG GTTCTCCCTC ACCACCCTCC
451   GTGACTTCGG GATGGGGAAA CAGGGCAATG AGCAGCGGAT CCAGAGGGAG
501   GCCCACTTCC TGCTGGAGGC ACTCAGGAAG ACCCATGGCC AGCCCTTTGA
5total (33%)

551   TCCCACCTTC CTCATCGGCT GCGCACCCTG CAATGTCATC TCCGACATCC
601   TCTTCCGCCA GCACTTTGAC TACAATGACA AGACCTGTCT GAGGTTAATG
651   AGCATGTTCA ATGAGAACTT CTACCTGCTC AGTACCGGCT GGATCCAGCT
701   TTATAATAAT TTCTCAGGCT ATCTACGCTA CCTGCCTGGA AGCCATAGGA
751   AACTAATGAA AAATATATCT GAAATAAAAG ATTATGCTTT AGAAAGAGTG
801   AAGGACCACC GGGATTCACT GGAGCCCAGC TGTCCTCGAG ATTTCACTGA
851   CACCCTGCTG ATGGAAATGG AGAAGGAAAA ATACAGTGCA GAACCTATAT
901   ACACCTTGGA CAACATTGCC GTGACCGTGG CCGACATGTT CTTTGCGGGG
951   ACAGAGACCA CCAGCACCAC CCTGAGATAC GGGCTCCTAA TTCTCATGAA
1001  ATACCCAGAG GTTGAAGAGA AACTTCATGA AGAAATTGAC AGGGTCATTG
1051  GTCCAAACAG AATCCCTGCC ATCAAGGACA GGCTGGACAT GCCCTACCTG
5total (63%)
```

Fig. 8

1101 GATGCCGTGG TACATGAGAT TCAGCGATTC ATCGACCTCA TTCCCTCCAA
1151 CCTGCCACAT GAAGCAACCC GGGACACAGT ATTCAGAGAC TACATCATCC
1201 CCAAGGGCAC AGTGGTAATT CCGACACTGG ACTCCGTCTT ATATGACAGC
1251 CAAGAATTCC CTGAGCCGGA GAAGTTTAAG CCAGAGCACT TTCTGAATGA
1301 AAACGGAAAG TTCAAGTACA GTGATCATTT CAAGGCATTT TCCGCAGGAA
1351 AGCGGGTGTG TGTCGGAGAG GGCCTGGCTC GCATGGAACT GTTCCTGTTC
1401 ATGGCTGCCA TCTTGCAGCA CTTTAACTTG AAATCTCTTG TTGACCCCAA
1451 GGATATCGAC CTCAGCCCCA TCGCGATTGG GTTTGCCAAG ATTCCCCCCC
1501 ATTACAAACT CTGTGTCATT CCCCGCTCAC AAGTGTGAGG GAGATGTGCT
1551 CTAAAGGCCC TGGTTCCTTG ATGCTGACCT GGAGGCCTCC TGTCCCCAGT
1601 GTCCCCACAG GGAGCGCAGC CCGGGCTCCA TAGGAAATCA AATGGGCCAG
Stotal (93%)

1651 TGAAGCTGCT TCCAGCCCAC ATCCTTCAGA TAGAATTTGA AAGCAAAGTC
1701 CAAAAAAGAT TTTGTACAAT CAATTAAAGT AAGTAAAGCC AAAAAAAAAA
1751 AAAAAAAAAA AAAAAAAA

Fig. 8 (Continued)

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                                                              Part of
    1 .................QPFDPTFLIGCAPCNVISDILFRQHFDYNDKTC  33 - SEQ ID No. 4
                       |||||||||||||||||||||||||||||||||       SEQ ID No. 5
  151 IQREAHFLLEALRKTHGQPFDPTFLIGCAPCNVISDILFRQHFDYNDKTC 200 - AB 000885

   34 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLMKNISEIKDYA  83
      ||||||||||||||||||||||||||||||||||||||||||||||||||
  201 LRLMSMFNENFYLLSTGWIQLYNNFSGYLRYLPGSHRKLMKNISEIKDYA 250

   84 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSAEPIYTLDNIAVTVADM 133
      ||||||||||||||||||||||||||||||||||||||||||||||||||
  251 LERVKDHRDSLEPSCPRDFTDTLLMEMEKEKYSAEPIYTLDNIAVTVADM 300

  134 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLD 183
      ||||||||||||||||||||||||||||||||||||||||||||||||| 
  301 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLV 350

  184 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTVVIPTLDSV 233
      |||||||||||||||||||||||||||||| |||||||||||||||||||
  351 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTDFRDYIIPKGTVVIPTLDSV 400

spice 9%
spice 9%
```

Fig. 9

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  1 ...GTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLD 47      - SEQ ID N0.6
       |||||||||||||||||||||||||||||||||||||||||||||||
301 FFAGTETTSTTLRYGLLILMKYPEVEEKLHEEIDRVIGPNRIPAIKDRLV 350     - AB000885

 48 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTVFRDYIIPKGTVVIPTLDSV 97
    |||||||||||||||||||||||||||||| |||||||||||||||||||
351 MPYLDAVVHEIQRFIDLIPSNLPHEATRDTDFRDYIIPKGTVVIPTLDSV 400

 98 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 147
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 LYDSQEFPEPEKFKPEHFLNENGKFKYSDHFKAFSAGKRVCVGEGLARME 450

148 LFLFMAAILQHFNLKSLVDPKDIDLSPITIGFAKIPPHYKLCVIPRSQV. 196
    |||||||||||||||||||||||||||| ||||||||||||||||||||
451 LFLFMAAILQHFNLKSLVDPKDIDLSPIAIGFAKIPPHYKLCVIPRSQV* 500
```

Fig. 10